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Enhancing the nutritional profile of regular wheat bread while maintaining technological quality and adequate sensory attributes†

Andrea Hoehnel,[a] Jürgen Bez,[b] Iben Lykke Petersen,[c] Ryszard Amarowicz,[d] Jerzy Juśkiewicz,[d] Elke K. Arendt,[*a,e] and Emanuele Zannini [a]

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Plant proteins, and legume proteins in particular, have become the centre of attention moving towards a more sustainable and, therefore, more plant-based human diet. Especially hybrid products, containing wheat and legume proteins, promise a balanced amino acid composition and an upgraded nutritional value of both protein sources. This study investigates a high-protein hybrid bread (HPHB) formulation, where wheat flour was partially replaced by high-protein ingredients from faba bean, carob and gluten. In addition to a detailed characterisation of technological quality and sensory profile, also the formulation's nutritional value was examined in comparison to regular wheat bread. Therefore, macronutrient composition, antioxidant potential, amino acid profile and contents of antinutritional compounds were analysed. Furthermore, protein digestibility was determined in an *in vitro* model and *in vivo*. Dough analysis revealed significant differences of the HPHB formulation compared to regular wheat dough. However, results obtained for bread quality characteristics prove HPHB to be equal to regular wheat bread and sensory results and the determined sensory attributes suggest high consumer acceptance. Nutritional analyses of HPHB showed a more favourable macronutrient composition in comparison to regular wheat bread; as well as low contents of antinutritional compounds and high antioxidant potential linked to high levels of phenolics. Also an improved amino acid profile, increased nitrogen utilisation rate (by 69 %) and higher protein efficiency ratio were determined, which are associated with enhanced protein quality. This suggests HPHB, and similar formulations of its kind, as a valuable and healthy food choice, which can contribute to adequate protein supply in predominantly plant-based diets.

1 Introduction

Protein from plant sources, next to other trends like digestive health and good carbs/bad carbs, is currently one of the most popular and important trends in the food sector.[1] One of the reasons for that is an increasing awareness amongst consumers, authorities and industry of the need to create a more sustainable food system considering planetary boundaries.[2,3] According to many recent reports, this requires a shift to a predominantly plant-based human diet.[2,4] Since we are also facing a growing world population, with a prospect of about 10 billion by 2050,[2] research plays a key role in finding ways to provide high-quality protein from plant sources to cover future protein needs. Even though it is known that current protein consumption exceeds the average daily requirement in many parts of the world, this is usually linked to high intakes of animal protein and necessary changes in the food system and human diet are likely to pose a challenge to sufficient protein supply in the future.[2,4] In many cases, the overconsumption of protein is associated with a general overconsumption of food and energy intakes exceeding recommended levels[4] and does not reflect an overconsumption of protein relative to other macronutrients. Furthermore, recommendations for daily protein intakes are based on high-quality protein. When large amounts of protein of lower quality are consumed, intakes might need to be increased in order to meet the body's amino acid requirements.[5] Apart from sustainability considerations, dietary

[a] *University College Cork, School of Food and Nutritional Sciences, College Road, Ireland. Tel: +353 21 490 2064; E-mail: e.arendt@ucc.ie*
[b] *Fraunhofer Institute for Process Engineering and Packaging, 85354 Freising, Germany.*
[c] *Department of Food Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg C., Denmark.*
[d] *Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima, St. 10, 10-748 Olsztyn, Poland.*
[e] *APC Microbiome Ireland, Cork, Ireland.*

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recommendations advice a reduction of animal protein intake in favour of increased plant protein consumption for a healthy diet. Many reported adverse effects of high protein intake are largely related to proteins from animal sources and the co-intake of sodium, nitrate, nitrite and saturated fatty acids when red meat or dairy products are consumed.[2,4,6] Also an overconsumption of food carbohydrates, especially refined carbohydrates, has been reported for a large number of countries and associated with increased health risks.[7] Research concerning new alternative plant protein sources is mostly focused on legumes. Due to their ability to grow in a variety of different climates and to fix nitrogen in the soil, they are particularly promising for a local crop cultivation, a considerably reduced use of fertilisers and a food production with a lower carbon and water footprint.[3,8,9] Legumes are naturally rich in protein, which contains high amounts of the essential amino acid (AA) lysine but lacks sulphur-containing amino acids (SAAs).[8,10,11] This makes legumes particularly interesting for the complementation of cereal based diets, since cereals have little lysine and higher amounts of SAAs.[12,13] Efforts have been made to combine both protein sources in "hybrid products" containing cereals and legumes and especially wheat bread has proven a suitable cereal matrix for the incorporation of legume protein ingredients.[14] Ideal bread should have a lower glycaemic index than regular white bread, be an important source of proteins, and contain tolerated dietary fibre, vitamins, magnesium, trace elements and antioxidants.[15,16] Jenkins *et al.*[7] state that, in the context of decreased physical activity in our population, foods should possess nutritional density rather than nutrient density. This means that the intake of essential nutrients (macro and micro) per calorie will need to increase in order to meet requirements at lower caloric intake levels. Legumes are rich in micronutrients and compounds with antioxidant activity, which could help to enhance the nutritional value of wheat bread.[14,17,18] Also a lowered glycaemic load, increased protein content and improved protein quality could be achieved by the fortification of wheat bread with legume proteins. Numerous research articles have investigated the effects of legume ingredients, from faba bean (*Vicia faba*) and carob (*Ceratonia siliqua*) seeds in particular, on both the technological as well as nutritional quality of breads.[14,19–23] However, many of these publications report inferior technological and sensory characteristics in favour of increased nutritional quality. Additionally, there are concerns regarding antinutritional compounds (ANCs) originating from legumes such as trypsin inhibitors, tannins, lectins and the pyrimidine glycosides vicine and convicine. Trypsin inhibitors, which can negatively impact protein digestibility, are present in many plants but are particularly important in legumes.[24,25] Vicine and convicine are mainly found in faba beans and can trigger adverse physical conditions like favism.[26–28] This leads to a low popularity of legume ingredients and cereal/legume hybrid products.[29] Next to an enhanced nutritional value, adequate technological quality and sensory properties are essential for a high consumer acceptance of such products and for an acceleration of the protein transition in our diet. This is why this study proposes and fully characterises a new bread formulation, which was designed to match the technological quality of regular wheat bread, but promises an improved nutritional profile with a higher protein content and higher protein quality in particular. Therefore, plant-based high-protein ingredients (HPIs) from faba beans, carob and wheat, selected based on findings by Hoehnel *et al.*[30], were incorporated in a regular wheat bread. This high-protein hybrid bread (HPHB) formulation was evaluated regarding technological, nutritional and sensory characteristics using regular wheat bread as a reference (RWB). The HPHB formulation, containing a dry-processed faba bean HPI as its main source of non-wheat protein, also promises improved sustainability;[31] especially when compared to other high-protein bread formulations that are commercially available. These often contain dairy ingredients as non-wheat protein source. Vogelsang-O'Dwyer *et al.*[32] reported a life cycle assessment (LCA) of the dry-processed faba bean HPI used in this study, which confirmed reduced use of land and water resources as well as lower impact on climate change (carbon footprint) and aquatic eutrophication in comparison to cow's milk powder. This makes HPHB and formulations of its kind even more promising to partially cover protein needs of future predominantly plant-based diets.

2 Materials and Methods

2.1 Materials

Three high-protein ingredients (HPIs) were applied in the high-protein hybrid bread (HPHB) formulation. Faba bean flour (protein content 61.25 %DM, fat 3.81 %DM, ash 5.43 %DM, fibre 0.35 %DM, carbohydrates by difference 29.17 %DM, total starch 7.77 %DM;[30] obtained by dry fractionation), which was experimentally produced and provided by Fraunhofer Institute IVV, Freising, Germany; carob germ flour (protein content 55.04 %DM, fat 0.20 %DM, ash 7.04 %DM, fibre 17.67 %DM, carbohydrates by difference 20.05 %DM, total starch < 0.2 %DM;[30] GRINDSTED VEG PRO S1) from Danisco, UK and vital gluten (protein content 72.38 %DM, fat 0.72 %DM, ash 0.87 %DM, fibre < 0.1 %DM, carbohydrates by difference 15.31 %DM, total starch 4.95 %DM;[30] NUTRALYS W) from Roquette, France. Wheat flour was supplied by Whitworth Bros Ltd, UK; dry yeast by Puratos, Belgium; salt by Glacia British Salt Ltd, UK; sugar (granulated Irish sugar) by Nordzucker (Ireland) Ltd, Ireland; psyllium (VITACEL P95) by J. Rettenmaier & Söhne, Germany; vegetable oil by Musgrave, Ireland; and xylanase (Biobake 715) by Kerry Group, Ireland. For *in vivo* digestibility trials, the following ingredients were used for the preparation of diets: casein (C) from Lacpol Co., Poland; soya protein isolate (SPI) ISOPRO 900 HI characterised as non-GMO protein isolate from EDMIR-POL Co., Poland; soya flour (SF) SOPRO TB 200 from EDMIR-POL Co., Poland; α-cellulose (C8002) from Sigma-Aldrich, Missouri, USA; soya oil from ZPT Co., Poland; choline chloride from SIGMA, Poland; cholesterol from PPH Standard Co., Poland; sucrose from POCH SA Co., Poland; and corn starch from Avebe, The Netherlands. Enzymes for *in vitro* digestion trials were purchased from Sigma-Aldrich, Missouri, USA: Pepsin from porcine gastric mucosa; EC 3.4.23.1; P7000; 727 U/mg and pancreatin from porcine pancreas; 4 x USP; P1750. All other chemicals were also purchased from Sigma-Aldrich, Missouri, USA unless stated otherwise.

2.2 Technological Analysis

2.2.1 Flour Analysis

The properties of wheat flour (used for reference wheat bread) and the high-protein (HP) flour mix (used for HPHB) were analysed. The HP flour mix contained wheat flour, the three HPIs (faba bean flour, carob germ flour, gluten) and psyllium in ratios according to HPHB formulation (Table 1). The moisture content of the HP flour mix was calculated considering the moisture determined for each single ingredient. GlutoPeak test - Gluten-aggregation properties of wheat flour and the HP flour mix were investigated following the method previously described by Hoehnel *et al.* [30] using the GlutoPeak device (Brabender GmbH and Co KG, Duisburg, Germany). In brief, high shear was applied to a flour/water slurry (50:50 ratio, adjusted when moisture of flour differed from 14 %). The device was operated at a paddle speed of 2750 rpm and temperature of 36 °C; torque was recorded over time. Variables Torque maximum (TM, expressed in Brabender units BU) and Peak Maximum Time (PMT, expressed in s) were obtained from the curves. Rapid visco analysis - Examination of pasting behaviour using Rapid Visco Analysis (RVA Super 3, Newport Scientific, Warriewood, Australia) was performed according to AACC 76-21.02. The following heating profile was applied: equilibration at 50 °C for 1 min, heating to 95 °C at 0.2 °C/s, holding at 95 °C for 162 s, cooling to 50 °C at 0.2 °C/s, maintaining at 50 °C for 120 s. The variables peak viscosity (PV), setback and final viscosity (FV) were obtained from the viscograms.

2.2.2 Recipe Adaptation and Bread Production

Bread samples were prepared according to the formulations in Table 1. The HPHB formulation contains HPIs (faba bean flour, carob germ flour, gluten) and was designed to match the technological quality of the reference wheat bread (RWB). A series of preliminary trials (data not shown) based on the results presented by Hoehnel *et al.* [30] led to the establishment of the HPHB formulation. A total of 28 different recipes were screened to select a combination of HPIs and to optimise their relative ratios for favourable technological characteristics. Furthermore, the introduction and optimal addition levels of the functional ingredients psyllium, sugar and xylanase were investigated as part of the screening to achieve adequate dough handling characteristics and quality of the end product. For both formulations, the straight dough method was applied. Yeast was activated by dissolving in 30 °C tap water for 10 min. The obtained yeast suspension was added to the remaining, previously weighed ingredients. A total amount of 3600 g dough was prepared. Mixing conditions were the following: RWB - MACPAN MX 10 spiral mixer (MACPAN SNC, Italy) at speed 1 for 6.5 min and speed 2 for 5 min; HPHB - Hobart A200N mixer (Hobart Manufacturing, UK), equipped with hook attachment, at speed 1 for 2 min and speed 2 for 7.5 min. After covering the dough and leaving it to rest for 5 min, it was divided into 7 pieces of 450 g $\pm$ 1 g. The pieces where moulded, put into baking tins and proofed for 90 min at 75 % humidity and 35 °C (KOMA BV Sunriser, Reormond, the Netherlands). Baking was performed in deck ovens (MIWE Condo, Arnstein, Germany) at 220/230 °C top/bottom temperature for 35 min with open draft throughout the whole baking process. The baking chamber was steamed with 400 mL prior to loading. After baking, breads were removed from tins and left to cool down for 2 h at ambient temperature. The results were obtained from three independently performed baking trials.

2.2.3 Dough Analysis

Doughs for determination of dough properties were prepared as described in section 2.2.2. Rheofermentometer - Formation and retention of gas in the fermenting doughs was analysed using a Rheofermentometer F3 (Chopin, France). A dough piece (300 g) was placed into the sample container and a weight constraint of 1.5 kg was applied. The dough fermentation was monitored for 3 h at a temperature of 35 °C (matching the proofing temperature used during bread production). The fermentation performance of the doughs was evaluated by the following variables obtained from the generated curves: Total gas volume produced (V_{total}), volume of CO_2 lost (V_{lost}) and volume of gas retained (V_{ret}) from gaseous release curves; and maximum height of dough development (H_M) from dough development curves. Large deformation properties - Extensibility (expressed in mm) and resistance to extension (expressed in g) of the doughs were measured by a texture analyser (TA-XT plus, Stable Micro Systems, Surrey, UK) equipped with a 5 kg load cell and a Kieffer dough and gluten extensibility rig (test settings: pre-test speed 2 mm/s, test speed 3.3 mm/s, post-test speed 10.0 mm/s, trigger force 5 g). The uniaxial extension test was performed after a dough resting time of 20 min (room temperature) inside the dough strip mould. Ten intact strips of dough were measured from each of three batches per formulation.

2.2.4 Bread Quality Analysis

Specific volume (SV) was measured with a Volscan Profiler (Stable Micro Systems, Surrey, UK) of 6 loaves per batch. For analysis of crumb structure and hardness, three slices (20 mm) were cut out of the middle of each of 2 bread loaves. A C-Cell Imaging System (Calibre Control International Ltd, UK) was used to capture images of slices and to determine the variables: number of cells, area of cells and slice brightness. Crumb hardness was analysed with a texture analyser (TA-XT2i, Stable Micro Systems, Surrey, UK) equipped with a 25 kg load cell. A 35 mm cylindrical probe was used to compress the centre of the slice to 40 % of its height as part of a texture profile analysis (TPA): test speed 5 mm/s, post-test speed 10 mm/s, trigger force 0.05 N, waiting time between compressions 5 s. TPA of bread slices was repeated on day 2 and day 5 after baking to monitor bread staling (whole loaves were stored in plastic bags at ambient temperature in the bakery and sliced immediately before the measurement). Lightness of crust (L^*crust) and crumb (L^*crumb) was measured by a Colorimeter CR-400 (Konica Minolta, Japan) using the CIE $L^*a^*b^*$ colour space.

2.2.5 Scanning Electron Microscopy

Bread crumb was separated from crust, cut into small cubes, frozen at -80 °C and freeze-dried. The dry crumb was further crushed into small fragments which were mounted onto plain aluminium stubs with double-sided carbon adhesive tape. After

Table 1 Recipe for RWB and HPHB

Ingredient	Reference wheat bread		High-protein hybrid bread	
	% based on flour	% based on recipe	% based on flour	% based on recipe
Wheat flour	100.0	59.70	82.5[×]	47.22[×]
Faba bean flour	-	-	10.0[×]	5.72[×]
Carob germ flour	-	-	5.0[×]	2.86[×]
Gluten	-	-	2.5[×]	1.43[×]
Psyllium	-	-	2.0[×]	1.14[×]
Sugar	-	-	1.0	0.57
Baker's yeast	2.0	1.19	2.0	1.14
NaCl	2.0	1.19	2.0	1.14
Oil	1.0	0.60	1.0	0.57
Xylanase	-	-	0.0060	0.0034
Water	62.5	37.31	66.70	38.18
Total	167.5	100.00	174.7	100.00

[×] Ingredients are included in HP flour mix

coating with a 5 nm gold-palladium (80:20) layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEMcoating system, England), they were examined under high vacuum with a JOEL scanning electron microscope (SEM) type 5510 (JOEL Technics Ltd., Tokyo, Japan). Images were acquired at a constant accelerating voltage of 5 kV.

2.3 Nutritional Analysis

Analysis of nutritional characteristics of the bread formulations was performed on freeze-dried (according to the procedure described in section 2.2.5) and subsequently milled (laboratory disc mill; Bühler, Brauchschweig, Germany) samples of bread crumb. Results are expressed as contents in fresh bread considering the moisture of freeze-dried and fresh bread crumb unless stated otherwise.

2.3.1 Compositional Analysis

The analysis of the following compositional data was performed by Concept Life Science Ltd., UK based on the indicated validated methods: energy (calculated considering protein, fat, available carbohydrates and fibre), protein (Dumas method, modified after AOAC 1977.992.15; nitrogen-to-protein conversion factor 6.25), ash (removal of organic matter by oxidation at 550 °C, based on ISO 936:1998), fat (low resolution proton nuclear magnetic resonance (NMR), based on MQC-23-35 Oxford Instruments application note), fatty acid profile (GC-FID of fatty acid methyl esters; triglyceride conversion factor 0.956), total dietary fibre (TDF) (gravimetric method, based on AOAC 991.43), sodium (flame photometry after removal of organic matter). Moisture was determined by air-oven method at 130 °C until constant mass was reached. Total starch content was analysed using the enzyme kit K-TSTA supplied by Megazyme, Ireland. Mono-, di- and oligosaccharides were extracted from the freeze-dried product powders as follows: 15 mL of 80/20 (v/v) ethanol/ultrapure water (80% EtOH), which was heated to 55 ± 5 °C, were added to 2 g of sample. The mixture was vortexed until the powder was suspended and then subjected to sonication (extraction step 1) utilising a BANDELIN Sonoplus HD 3100 homogenizer (Berlin, Germany) equipped with an MS73 microtip, operated twice for 15 s at 75 °amplitude. Hereupon, the sample was centrifuged at 1800 g for 10 min and the supernatant transferred to another test tube for further processing. Sonication and centrifugation were repeated (extraction step 2) after adding another 15 mL 80% EtOH (at 55 ± 5 °C) to the pellet. The supernatants of both extraction steps were pooled and concentrated using a vacuum centrifuge system (Scanvac Scan Speed 32 with Scanvac VacSafe 15, Labogene ApS, Lynge, Denmark) with the following settings: 2 h at 1500 rpm and 45 °C, followed by 1 h at 2000 rpm and 50 °C; average pressure 15 mbar. The concentrated extract was transferred into a 10 mL volumetric flask, which was filled up with ultrapure water containing 50 mg/L NaN_3, and filtered through syringe driven polyamide filters (Chro-mafil AO-20/25, pore size: 0.20 μm, Machery-Nagel, Düren, Germany). Samples were extracted in duplicates and quantification of the sugars was performed according to the method described by Ispiryan *et al.* [33] using a Dionex ICS-5000$^+$ system (Sunnyvale, CA) equipped with an electrochemical detector.

2.3.2 Amino Acid Analysis

Determination of protein amino acid composition was carried out by Mérieux NutriSciences CHELAB S.r.l., Italy based on ionic chromatography with postcolumn ninhydrin derivatisation (fluorescence detection; UV detection for tryptophan) after adequate extraction and protein hydrolysis (separate hydrolysis procedures for the determination of tryptophan, sulphur-containing AA and remaining AA).

2.3.3 *In vitro* Protein Digestion

A previously described static multi-step method for *in vitro* protein digestibility (IVPD) [34,35] was used to simulate gastro-pancreatic protein digestion. In short, sample amounts containing 50 ± 1 mg protein were weighed in and enzymatic hydrolysis was started: pepsin digestion at 37 °C and pH 1-2 (1 h) followed by sequential pancreatin digestion at 37 °C and pH 7-8 (short-term: +1 h; medium-term: +3 h; long-term: +24 h). Ratios between enzyme and substrate (w/w) were kept constant at 1:50 (pepsin stage) and 1:10 (pancreatin stages). IVPD in % was determined using a trinitrobenzenesulfonic acid (TNBS) assay. Results are expressed as the concentration of free α-amino groups in samples in relation to an alanine standard solution representing 100 % protein digestibility.

2.3.4 *In vivo* Nitrogen Balance

The animal protocol used in this study was approved by the local institutional Animal Care and Use Committee (Olsztyn, Poland) and the study was performed in accordance with EU Directive 2010/63/EU for animal experiments. The assessment was conducted on growing male Wistar rats weighing 173.2 g. The rats were randomly divided into groups of seven animals. All animals were housed individually over 14 days in metabolic cages with free access to water and the experimental diets (Table 2). The selection of the animals and their maintenance over the 14-day experiment followed common regulations. The environment was controlled with a 12 h light-dark cycle, a temperature of 22±1 °C, relative humidity of 45-65% and 20 air changes per hour. For experimental feeding the following diets were used: a standard control diet based on casein (C) as the main protein source (supplemented with 0.2% DL-methionine), a second control diet based on soya protein isolate (SPI, without any supplementation), a third control diet based on soya flour (SF, without any supplementation) and the experimental diets containing RWB and HPHB. All experimental diets were a modification of the AIN-93G diet for laboratory rodents recommended by the American Institute of Nutrition;[36] the dietary protein level was lowered to approx. 11% to measure the protein digestibility and utilisation rate. During the study, nitrogen (N) digestibility and utilisation tests (balance tests) were carried out. After a 9-day preliminary period, faeces and urine were thoroughly collected for 5 d from all rats (kept in balance cages; Tecniplast Spa, Buguggiate, Italy). The total N content of each diet as well as each faecal and urinal sample (collected in the balance period) was analysed in duplicate (AOAC 979.09). The rats from each diet group were additionally monitored for body-weight (BW) gains (recording BWs at the beginning and end of the study) and diet intake (daily record), which enabled calculation of the protein efficiency ratio (PER). All physiological measurements were carried out for each animal separately (n = 7 per diet group).

Table 2 Composition of diets for *in vivo* nitrogen balance trials, values given in % of diet

Component of diet	C	SPI	SF	RWB	HPHB
Casein	**11.15**				
DL-Methionine	**0.20**				
Soya protein isolate		**10.80**			
Soya flour			**19.69**		
Reference wheat bread				**67.79**	
High-protein hybrid bread					**43.85**
Cellulose	8.00	8.00	8.00	8.00	8.00
Soya oil	8.00	8.00	8.00	8.00	8.00
Mineral mix[1]	3.50	3.50	3.50	3.50	3.50
Vitamin mix[2]	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.20	0.20	0.20	0.20	0.20
Cholesterol	0.30	0.30	0.30	0.30	0.30
Sucrose	5.00	5.00	5.00	5.00	5.00
Corn starch	62.65	62.29	54.31	6.21	30.15

[1] AIN-93G-MX: mineral mixture as specified by Reeves [36] (1997)
[2] AIN-93G-VX: vitamin mixture as specified Reeves [36] (1997)

2.3.5 Antinutritional Compounds

Trypsin inhibitors were extracted from the lyophilised product powders by adding 2.5 mL sodium acetate buffer (0.1 M, pH4.9) to 350 mg sample and homogenising the mixture for 2 min using an Ultra Turrax. After centrifugation for 5 min at 3000 g (EBA 12 Centrifuge; Hettich Zentrifugen, Tuttlingen, DE), the supernatant was transferred to a new test tube and the extraction procedure was repeated with the same conditions with the pellet. Both supernatants were pooled, stored in the fridge overnight and centrifuged again (5 min, 3000 g) immediately before trypsin inhibitor activity (TIA) analysis. TIA was determined following the method described by Joehnke *et al.* [34] with some modifications. In brief, TIA levels were measured against a trypsin solution (stock concentration 0.1 mg/mL). A solution of N-α-benzoyl-L-arginine-4-nitroanilide (L-BAPA) with 0.22 mg/mL was used as substrate. Spectrometric quantification was performed at 410 nm and based on a molar extinction coefficient of the reaction product (4-nitroaniline) of 8800 $M^{-1}cm^{-1}$. One trypsin inhibitor unit (TIU) is defined as the amount of inhibitor required to reduce the trypsin activity by one unit. One trypsin activity unit (TAU) is defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol L-BAPA into 4-nitroaniline within 1 min at pH 8.2 and 37 °C. Contents of vicine and convicine were determined after an extraction of 500 mg of sample with boiling methanol as described by Petersen *et al.* [37]. Quantification was achieved using micellar electrokinetic capillary chromatography as reported by Bjergegaard *et al.* [38] and with vicine as external standard.

2.3.6 Antioxidant Potential

Extraction - Phenolic compounds were extracted from the product powders using 80/20 (v/v) methanol/water (80% MeOH), at a solid to solvent ratio of 1:10 (w/v), for 15 min at 50 °C as described by Amarowicz *et al.* [39]. The extraction was repeated twice, the supernatants were filtered and pooled, and the methanol was evaporated under vacuum with a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). The remaining aqueous extract was lyophilised. Total phenolic content (TPC) - TPC of phenolic extracts was determined using Folin-Ciocalteu's phenol reagent following a method described by Amarowicz and Raab [40]. The results were expressed as mg catechin equivalent. Trolox equivalent antioxidant capacity (TEAC) - TEAC was determined according to the method reported byRe *et al.* [41]. In brief, a $ABTS^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) solution was prepared by mixing an aqueous ABTS stock solution with 2.45 mM (final concentration) sodium persulfate. This mixture was shaken for 12-16 h at room temperature in the dark until a stable oxidative state was reached. The $ABTS^{\bullet+}$ stock solution was diluted with methanol to an absorbance of 0.720 at 734 nm for subsequent analysis. For the spectrophotometric assay, 2 mL of the diluted $ABTS^{\bullet+}$ solution were mixed with 20 μl of reconstituted phenolic extract (10 mg/mL in methanol); absorbance was determined at 734 nm at 37 °C for 10 min. A calibration curve was generated using a Trolox standard and the results were expressed as μmol Trolox equivalent. Ferric-reducing antioxidant power (FRAP) - FRAP assay was performed as described by Benzie and Strain [42]. The FRAP value was calculated and expressed as μmol Fe^{2+} using a Fe^{2+} calibration curve. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay - The radical scavenging effect of the phenolic extracts was measured as described in Amarowicz *et al.* [43]. A

Table 3 Sensory attributes and extremes of intensity scales used for QDA of breads

Attribute	Definition	Extremes
Odour		
Sweet	Odour characteristic of sweet buns produced from wheat flour	None - very intense
Acidulous	Odour characteristic of fermented products (e.g. vinegar, yoghurt)	None - very intense
Appearance		
Beige colour	Crumb colour intensity	Light - dark
Pore size	Visual impression of bread crumb porosity	Small - big
Pore distribution	Regularity of pore distribution in the crumb	Irregular - regular
Texture (manual)		
Elasticity	The extent to which bread crumb returns to its original shape when stretched	Low - high
Texture (oral)		
Chewiness	Extent of chewing necessary to prepare food for swallowing	Low - high
Adhesiveness	Degree of adhesiveness when chewing the food 10 times	Low - high
Moisture	Moisture released by the food after 10 chews	Low - high
Taste		
Rye-wheat bread	Aroma characteristics of commercial rye-wheat bread (retronasal)	None - very intense
Salty	Taste characteristic of NaCl (1 % in water)	None - very intense
Acidulous	Taste characteristics of citric acid (1 % in water)	None - very intense
Aftertaste	Lingering sensation after swallowing the sample	None - very intense
Overall quality	Conclusive evaluation of all attributes and their harmonic balance	Bad - very good

methanolic solution (0.1 mL), containing 0.02-0.10 mg of extract, was mixed with 2 mL of deionised water, and was then added to a methanolic solution of DPPH· (1 mM, 0.25 mL). The mixture was vortexed for 1 min and left to stand at room temperature for 20 min. the absorbance of the solution was measured at 517 nm. The results were expressed as half maximal effective concentration (EC_{50}) of the phenolic extract that scavenged 50% of DPPH radicals.

2.4 Sensory Analysis

Descriptive sensory profiling (quantitative descriptive analysis – QDA) was carried out in order to characterise the bread samples using an expert panel (n=8). The QDA procedure used in the study was in accordance with the standard ISO 13299:2016. Panellists with appropriate methodological preparation and experience in sensory profiling were selected, trained and monitored following ISO 13299:2016. Before the sensory analysis, the panellists' performance was evaluated using three parameters – repeatability, discrimination ability and homogeneity by applying analysis of variance. Before the sensory analysis, a 28-hour panel training was conducted on various bread samples, including bread from the local supermarkets, with the aim to familiarise the sensory panel with innovative bread samples and their features. A list of sensory attributes was created. Initially, panellists chose characteristics describing the samples individually, followed by a joint agreement on distinguishing attributes and their descriptions (see Table 3). A continuous scale (10 cm long) with the extremes specified in Table 3 was used. Sensory evaluation was carried out in three independent sessions.

2.5 Statistical Analysis

All measurements were performed in triplicate unless stated otherwise. Data analysis was carried out using RStudio, version 1.2.1335 with R version 3.6.1 (RStudio Inc, USA; R Core Team, r-project). One-way analysis of variance (ANOVA) with post-hoc pairwise Tukey's test was used to show significant differences ($p < 0.05$). When available, values are given as the mean $\pm$ standard deviation or uncertainty (amino acid profile).

3 Results and Discussion

3.1 Technological Characteristics

3.1.1 Flour and Dough Properties

The properties of flours and doughs used for breadmaking have a high impact on the quality of bread products. In addition to the ability to form a stable gluten-network, rheological characteristics such as pasting behaviour, dough extensibility and the dough's proofing performance determine flour and dough quality. Gluten-aggregation and pasting behaviour were evaluated for RWB based on wheat flour and for HPHB based on HP flour mix (Table 1). The aim was to compare measurements, which are commonly performed to determine baking quality of flours, for the two formulations in this study. It was decided to include not only the HPIs in the HP flour mix for flour analyses, but also psyllium, which was expected to have a high impact on rheological properties. Sugar and xylanase were shown to have no significant effect on the performance of the HP flour mix in these tests (preliminary trials, data not shown) and were left out. The GlutoPeak test revealed striking differences in gluten-aggregation properties of the two flours. The variables obtained from the curves are presented in Table 4. Wheat flour exhibits with 68 BU a significantly higher TM than HP flour mix (64 BU), but PMT was detected 14 s earlier for HP flour mix (46 s) than for wheat flour (60 s). When pure wheat flours are measured, a general trend towards earlier and higher gluten peaks for stronger flours with higher gluten contents and/or higher gluten quality has been reported in literature.[44–46] The gluten content in HP flour mix (calculated based on composition of ingredients and an average gluten content in wheat flour protein of 80 %) is about 0.5 % lower than in wheat flour, which could explain the slightly lower TM detected for HP flour mix. However, Hoehnel *et al.*[30] showed that the partial replacement of wheat flour by HPIs leads to complex changes in the gluten-aggregation profiles, which do not follow this general trend. Therefore, a comparison of gluten-aggregation profiles in addition to TM and PMT (or other variables obtained from the

Fig. 1 Flour properties of wheat flour and HP flour mix: (**A**) Gluten-aggregation profiles obtained by GlutoPeak test; (**B**) Viscograms obtained from rapid visco analysis describing pasting behaviour of RWB and HPHB with black line representing the applied temperature profile. Dashed curves represent standard deviation.

curves) is required (see Figure 1). The profile of wheat flour follows the typical sequence of initial torque increase, equilibrium plateau, rapid torque increase, peak maximum and torque decrease due to breakdown of gluten-network. The HP flour mix shows no pronounced equilibrium plateau and the torque increases rapidly towards its maximum right in the beginning of the measurement. Instead of a sharp peak with a rapid gluten breakdown, the peak is broad and torque remains high after its maximum. According to Goldstein *et al.*[47], a fast build-up of gluten-network followed by a sharp peak and rapid breakdown is associated with weak flours. The profile of HP flour mix indicates a strong and stable gluten-network due to the broad gluten-peak and delayed gluten breakdown. This could be caused by a co-networking of gluten with non-wheat proteins from faba bean and carob as suggested by Hoehnel *et al.*[30]. The lack of equilibrium plateau and rapid torque increase at the start can be explained by the high water absorption of psyllium and gluten[48,49] resulting in a higher initial viscosity of the sample slurry. Table 4 shows variables describing the pasting behaviour of the flours.

Table 4 Flour properties of wheat flour (used for reference wheat bread) and HP flour mix (used for high-protein hybrid bread)

Variable	Wheat flour	HP flour mix
GlutoPeak		
Peak maximum time (PMT) [s]	60 ± 4^a	46 ± 2^b
Torque maximum (TM) [BU]	68 ± 1^a	64 ± 1^b
Rapid Visco Analyser		
Peak viscosity (PV) [cP]	2261 ± 9^a	1989 ± 17^b
Setback [cP]	1350 ± 19^b	1587 ± 59^a
Final viscosity (FV) [cP]	2607 ± 10^b	2756 ± 84^a

Means ± standard deviation with different letters in the same row were significantly different at $p < 0.05$.

The corresponding viscograms are displayed in Figure 1. The viscograms suggest a generally similar pasting behaviour of wheat flour and HP flour mix with only small discrepancies. However, significant differences have been detected for PV, setback and FV. The PV of HP flour mix is with 1989 cP lower than for wheat flour with 2261 cP. This can be attributed to the lower starch content in HP flour mix and, thus, less gelatinising starch, which has been previously observed in systems based on wheat flour[50] as well as systems based on rice flour.[51] The presence of psyllium in the HP flour mix is expected to increase viscosity of the sample due to its well-known high water absorption and gelling properties (at low temperatures as well as upon heating).[52,53] This might have partly compensated for the reduced viscosity owing to less starch. Hence, only a small difference in PV has been found. In contrast to a lower PV, HP flour mix exhibits higher FV and setback compared to wheat flour. Especially the setback expressed in relation to PV is remarkably high for HP flour mix (wheat flour: 59.7 %, HP flour mix: 79.8 %). A similar pattern was observed by Hoehnel *et al.*[30] in a flour blend containing 15 % faba bean flour. Since this ingredient contains a considerable amount of non-wheat starch, high setback and FV could be related to the retrogradation properties of faba bean starch. Dough analyses provide information on rheological and expansion properties of the formulations during proofing. Large deformation properties (Table 5) reveal a reduced extensibility and resistance to extension for the HPHB dough (13.04 mm and 0.475 N, respectively) compared to RWB (16.76 mm and 0.647 N, respectively). According to literature,[54] reduced resistance to extension as well as area under the curve are indicative of weaker doughs. However, also the shape of the curve (see Figure 2), and the ratio of resistance to extension and extensibility (R/E) in particular, seems im-

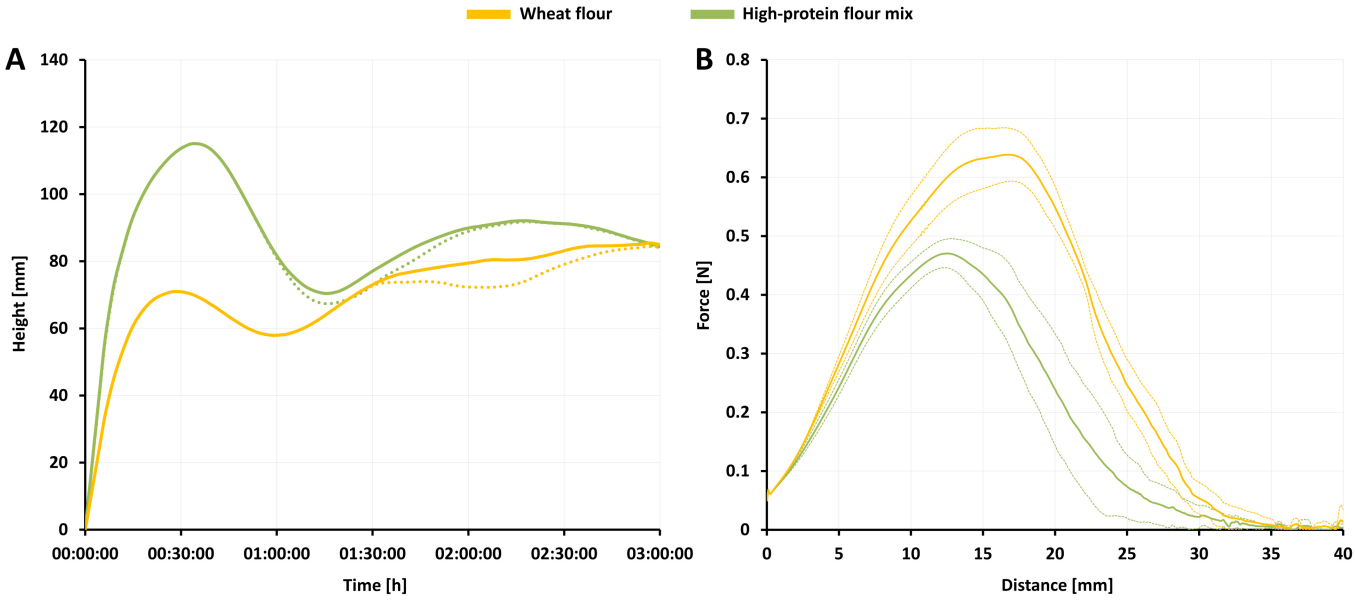

Fig. 2 Dough properties of RWB and HPHB: (**A**) Gas release curves obtained by Rheofermentometer measurements (dotted line represents gas retained in the dough); (**B**) Extensibility plots obtained Kieffer rig microextension tests (dashed curves represent standard deviation).

portant and provides information on the doughs' viscoelastic balance.[54] This ratio is with 0.039 N/mm for RWB and 0.036 N/mm for HPHB very similar for both formulations and suggests similar expansion properties. Variables describing the proofing performance of the doughs were obtained by Rheofermentometer measurements and are shown in Table 5. No significant difference was detected for dough development expressed as maximum height (H_M) with 67.3 mm for RWB and 61.6 mm for HPHB, which is in line with the similar expansion properties suggested by microextension tests. HPHB shows significantly higher total gas volume (V_{total}; 2449.7 mL) and retained gas volume (V_{ret}; 2416.3 mL) than RWB (1982.7 mL and 1924.3 mL, respectively). Also a tendency towards a lower lost gas volume (V_{lost}) for HP flour mix was observed. The gas release curves from Rheofermentometer measurements are displayed in Figure 2. The initial gas release is much more pronounced for HPHB than for RWB. This can be explained by the small amount (0.57 %) of added sugar in HPHB, which leads to higher initial yeast activity and gas production. The initial peak is followed by a temporary decline in gas release in both formulations. This represents the point where easily accessible sugars have been consumed by the yeast and further sugars are made available by enzymatic breakdown of starch and other polysaccharides present in the samples. Gas production at the start is the only remarkable difference in an otherwise very similar gas release profile throughout the measurement. Hence, the added sugar represents the main factor for the increased V_{total} of HPHB. Even though the difference observed in V_{lost} is not significant, also the curves suggest a tendency towards better gas retention of HPHB dough. This is in accordance with the findings of Courtin and Delcour.[55] They explained a positive effect of water-extractable arabinoxylans (AX) on gas retention of doughs related to a strengthening of liquid films surrounding CO_2 bubbles, thereby limiting gas diffusion. The psyllium in HPHB contains a considerable amount of AX, of which a small percentage is water-extractable.[56,57] Additionally, xylanase, which degrades water-unextractable AX (in HPHB from wheat flour[58] and psyllium[57]), increases the amount of water-extractable or solubilised AX present in the dough and their effect on gas retention properties.[55] Xylanase degradation of water-unextractable AX has also been reported to lead to a lowered water-binding capacity of AX and redistribution of water in favour of gluten, therefore facilitating gluten-network formation.[49,59] Wang *et al.*[60] discussed the formation of a secondary network based on AX with the ability to strengthen the gluten-network by entanglement and possibly the creation of diferulic bridges. This is in line with the stability of the gluten-network in HPHB and delayed breakdown indicated by GlutoPeak test results and represents an additional stabilising effect besides potential co-networking of gluten with non-wheat proteins.

Table 5 Dough properties of reference wheat bread formulation and high-protein hybrid bread formulation

Variable	RWB	HPHB
Kieffer rig extensibility		
Resistance to extension [N]	0.647 ± 0.059^a	0.475 ± 0.045^b
Extensibility [mm]	16.76 ± 1.25^a	13.04 ± 1.44^b
Rheofermentometer		
Dough development (H_M) [mm]	67.3 ± 5^a	61.6 ± 1^a
Total gas volume (V_{total}) [mL]	1982.7 ± 171.1^b	2449.7 ± 102.3^a
Volume of CO_2 lost (V_{lost}) [mL]	58.0 ± 30.5^a	33.0 ± 19.2^a
Volume of gas retained (V_{ret}) [mL]	1924.3 ± 192.1^b	2416.3 ± 103.0^a

Means $\pm$ standard deviation with different letters in the same row were significantly different at $p < 0.05$.

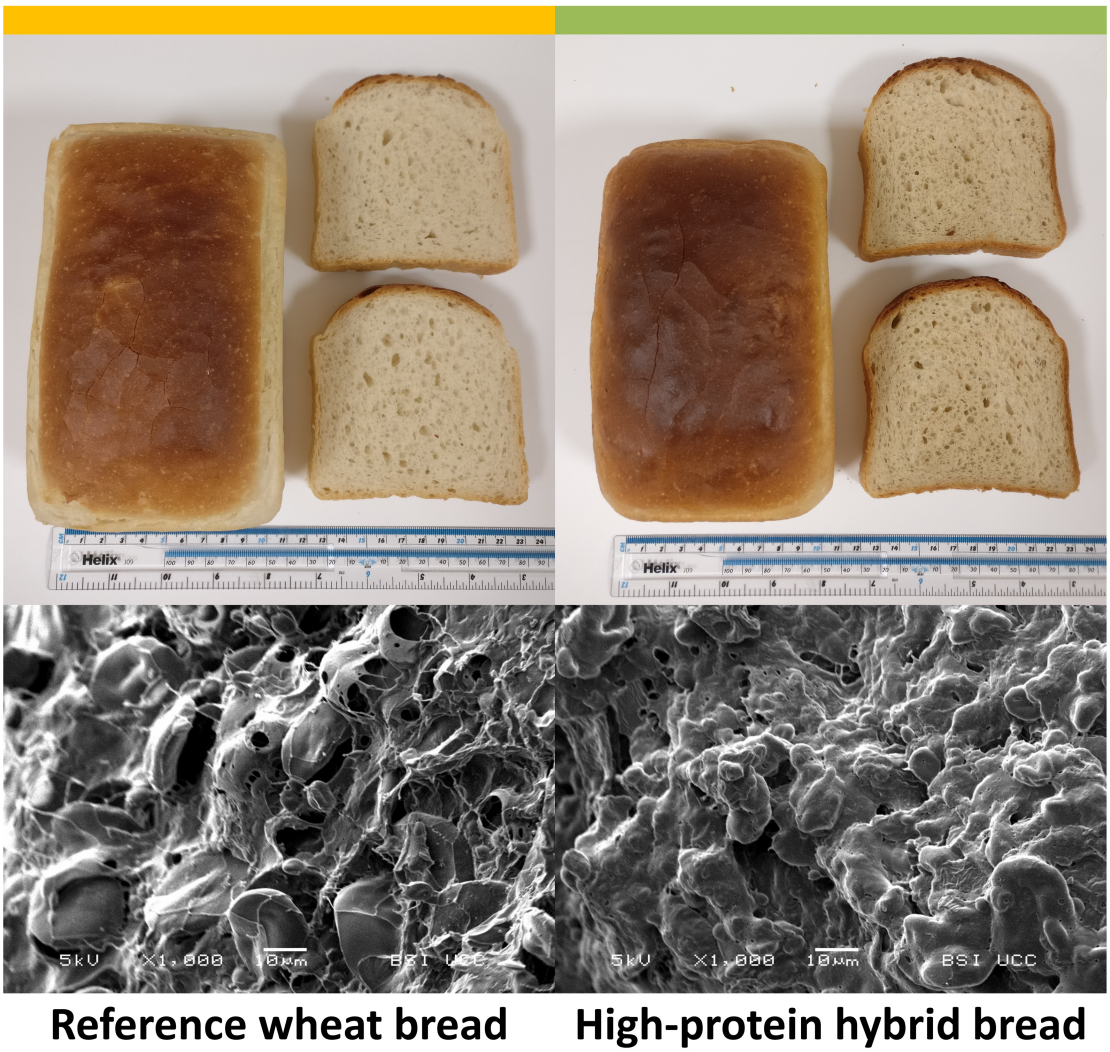

Fig. 3 Photographs and micrographs (obtained by SEM) of RWB and HPHB.

3.1.2 Bread Quality Characteristics

The breads produced from both formulations examined in this study are presented in Figure 3. A visual evaluation reveals little differences in loaf size and crumb structure between RWB and HPHB, but a considerably darker crust and crumb colour for HPHB. The results obtained for bread quality characteristics confirm this general observation and are reported in Table 6. No significant differences between RWB and HPHB have been detected regarding bake loss and SV. The initial crumb hardness on day 0 is with 6.98 N for HPHB slightly higher than for RWB (5.13 N). However, this can only be considered a small difference, especially when compared to previously reported increases in crumb hardness caused by the incorporation of legume ingredients in wheat bread.[22,30,61,62] Additionally, the crumb hardness measured on day 2 and day 5 does not show significant differences between RWB and HPHB. This indicates similar staling properties of both formulations, with a tendency towards less staling for HPHB. Staling rates calculated for day 2 are 1.42 for RWB and 0.78 for HPHB, which represents a by 45 % lower staling rate of HPHB. Staling rates calculated for day 5 are 2.08 and 1.31 for RWB and HPHB, respectively. Also here, HPHB shows a by 37 % lower staling rate. Recrystallysing starch is considered to be the main factor for staling of bread crumb.[63,64] Therefore, the decreased crumb staling in HPHB could be related to its lower starch content and, supposedly, a lower amount of gelatinised starch which

Table 6 Bread quality characteristics of reference wheat bread and high-protein hybrid bread

Variable	RWB	HPHB
Bake loss [%]	12.3 ± 0.6^a	11.9 ± 0.8^a
Specific volume (SV) [ml/g]	3.73 ± 0.07^a	3.75 ± 0.13^a
Hardness day 0 [N]	5.13 ± 0.43^b	6.98 ± 0.60^a
Hardness day 2 [N]	12.41 ± 1.43^a	12.41 ± 1.23^a
Hardness day 5 [N]	15.81 ± 0.85^a	16.15 ± 2.06^a
Number of cells	5009 ± 245^b	5563 ± 575^a
Cell area [%]	52.4 ± 0.3^a	51.7 ± 0.5^b
Slice brightness	137 ± 4^a	108 ± 3^b
Lightness of crumb (L^*crumb)	63.6 ± 2.2^a	60.4 ± 3.9^a
Lightness of crust (L^*crust)	41.9 ± 5.0^a	34.6 ± 2.9^b

Means $\pm$ standard deviation with different letters in the same row were significantly different at $p < 0.05$.

can recrystallise. Also AX and xylanases have been reported to decrease staling of wheat-based bread formulations.[65,66] The effect has been attributed to a competition for water and therefore a reduced swelling and gelatinisation of starch.[65,66] Maeda and Morita[67] observed reduced staling up to 3 days caused by both water-extractable and water-unextractable AX. While their study was focused on wheat AX, Czuchajowska *et al.*[68] also reported a reduced crumb hardness after 72 h when psyllium was incorporated in wheat bread. Specific volume and crumb hardness are generally accepted as the main indicators of bread quality. Therefore, the presented results confirm a technological quality of HPHB similar to RWB. The evaluation of crumb structure reveals small differences between the formulations. A slightly finer crumb structure was observed for HPHB indicated by a higher number of cells (5563) and smaller cell area (51.7 %) compared to RWB with a number of cells of 5009 and a cell area of 52.4 %. This can be related to the higher initial yeast activity and gas production in HPHB. Moulding of dough, in addition to shaping the dough pieces, leads to a division of gas cells produced prior to moulding (during mixing and dough rest).[69] In HPHB, more gas is produced before moulding and a higher number of small gas cells can be generated. Additionally, these gas cells are stabilised by water-extractable and solubilised AX as explained above, which can minimise the coalescence of gas cells as they expand during proofing and lead to a high number of cells in the final product. The higher number of cells and smaller cell area measured for HPHB could also be partially responsible for its slightly higher initial crumb hardness. Values obtained for crumb and crust colour (Table 5) confirm the visually perceivable differences between RWB and HPHB. Slice brightness (obtained by C-Cell imaging) is significantly lower for HPHB with 108 than for RWB with 137. This is in line with the lower lightness of crumb measured for HPHB. A big difference was observed in lightness of crust, which was significantly lower for HPHB (34.6) than for RWB (41.9). The darker crust of HPHB is likely related to its higher protein content and higher presence of reducing sugars (see Table 7), thus, an increased potential for Maillard reaction.[30,70] The micro-structure of the bread crumb of both formulations was captured by scanning electron microscopy (SEM). The resulting micrographs are displayed in Figure 3. While RWB shows a rather porous layer of gluten covering partly intact starch granules, HPHB has a thicker and more continuous layer. This might be due to the presence of non-wheat proteins from faba bean and carob on one hand and psyllium on the other hand. The fact that a very homogenous and continuous layer was formed, further supports the theory of a co-networking of gluten with non-wheat proteins and psyllium AX.

3.2 Nutritional Characteristics

3.2.1 Macronutrient Composition and Sugar Profile

Compositional analysis of both formulations was performed in order to evaluate changes in macronutrient composition caused by the partial replacement of wheat flour by plant-based HPIs in HPHB and addition of psyllium to the formulation (Table 7). The determined bread constituents include all items that are mandatory for nutritional food product labelling according to European food legislation (regulation (EU) No 1169/2011[71]). In addition, the sugar profile, total starch content and other important components of the samples were measured or calculated. Protein content and content of available carbohydrates represent the main differences in the macronutrient profile of RWB and HPHB. This is essentially caused by the replacement of wheat flour, which is high in starch (72.38 %DM), by HPIs with protein contents of 61.25 %DM (faba bean flour), 55.04 %DM (carob germ flour) and 83.11 %DM (gluten) and starch contents below 10 %DM (protein and starch contents of wheat flour and HPIs previously reported by Hoehnel *et al.*[30]). While the total energy level of the formulations is similar (RWB 211.6; HPHB 209.0), a shift from wheat starch to non-wheat protein characterises the macronutrient profile of HPHB. This shift is also evident when proteinE values (percentage of calories provided by protein) are compared. In contrast to RWB with 15.5 %E, the HPHB formulation reaches a proteinE of 24.8 %E and therefore qualifies for a "high in protein" nutritional claim in accordance with European food legislation (regulation (EC) No 1924/2006[72]), where a proteinE of 20 % is set as requirement. Bread is a staple food with global importance as source of dietary carbohydrates, protein and fibre.[15] However, within the past 200 years, the consumption of refined-carbohydrate products, including bakery products from refined wheat flour (white bread, white bagels, white buns), has substantially increased. At the same time, significantly less regular starchy foods like beans, lentils and wholegrain bakery products are consumed.[7] This is largely associated with generally bet-

Table 7 Composition of reference wheat bread and high-protein hybrid bread, contents expressed in % of the fresh bread unless stated otherwise

Component	RWB	HPHB
Moisture	45.74 ± 0.06	45.91 ± 0.29
Energy [kcal/100 g]	211.6	209.0
Protein	8.2	13.0
proteinE * [%E]	15.5	24.8
Ash	1.6	2.0
Fat	0.91	1.25
SFA	0.11	0.17
MUFA	0.26	0.36
PUFA	0.50	0.67
Total carbohydrates**	43.5	37.9
Total dietary fibre (TDF)	1.8	2.8
Available carbohydrates**	41.7	35.1
Total starch	36.1 ± 1.2	28.5 ± 0.6
Sodium	0.466	0.440
Sodium expressed as salt (NaCl)	1.16	1.10
Sum of mono- and disaccharides	1.21 ± 0.00	1.13 ± 0.02
Arabinose	<0.01	<0.01
Xylose	<0.01	<0.01
Galactose	0.01 ± 0.00	0.03 ± 0.00
Glucose	0.02 ± 0.00	0.03 ± 0.00
Fructose	0.02 ± 0.00	0.04 ± 0.00
Sucrose	0.01 ± 0.00	0.01 ± 0.00
Maltose	1.16 ± 0.00	1.02 ± 0.02
Maltotriose	0.03 ± 0.00	0.02 ± 0.00
Raffinose/Stachyose	<0.01	0.01 ± 0.00
Verbascose	<0.01	0.02 ± 0.00

Moisture, total starch and sugar profile: means ± standard deviation

* calculated based on energy content, protein content and 4 kcal/g protein

** calculated by difference

ter sensory characteristics of refined-carbohydrate products and potentially higher consumer acceptance due to their sweet taste when starch is rapidly digested by salivary amylase.[7,15] The main concern regarding this development is related to high glycaemic indices due to rapidly digestible starch .[2,7,15,73] High-glycaemic-load and high-glycaemic-index diets have been associated with elevated risk for diabetes, heart disease and certain types of cancer.[74–78] Due to its reduced content of available carbohydrates, HPHB is expected to have a lowered glycaemic load in comparison to RWB. Even a decreased glycaemic index could be expected, since psyllium has been reported to lower the glycaemic index of foods when added to conventional diets.[79,80] Holt *et al.*[81] found a significantly lowered blood glucose response of high-protein bread when they compared equal-energy portions of high-protein bread and regular white bread. Furthermore, an isocaloric replacement of refined starch or sugar by protein, like it is the case for HPHB compared to RWB, has been reported to reduce blood pressure and blood lipid concentrations.[2,82] Also the lack of fibre in refined-carbohydrate foods compared to wholegrain alternatives and legumes has been critically discussed.[7,15] Dietary fibre is associated with many health benefits and dietary recommendations advice a daily intake of 25 g or more for adults.[2,83] In the present study, HPHB contains with 2.8 % considerably more dietary fibre than RWB with 1.8 %. This is related to the incorporation of faba bean flour, carob germ flour and psyllium in HPHB, which represent ingredients with notable contents of both soluble and insoluble fibre.[30,57] Especially psyllium has been reported in literature as dietary fibre with beneficial effects regarding the risk of diabetes, obesity, high blood pressure and heart disease.[52] Apart from refined carbohydrates, also fat and salt (sodium cloride) are dietary components which are often critically discussed.[84–86] While HPHB contains with 0.440 % an amount of sodium similar to RWB (0.466 %), it has a slightly elevated fat content. However, this increase is mainly caused by higher contents of MUFA and PUFA, which are nutritionally more favourable than saturated fats.[2,84] Both formulations contain similar amounts of sugar (mono-and disaccharides) and their sugar profiles reveal little differences. They confirm that sucrose added in the recipe of HPHB is fully consumed during yeast fermentation, which was also evident in the results obtained from dough analyses. Slightly increased galactose and the presence of oligosaccharides like raffinose, stachyose and verbascose can be associated with high contents of galactooligosaccharides (GOS) reported for faba beans.[87] Slightly lower maltose and maltotriose levels in HPHB are potentially related to its lower starch content.

3.2.2 Amino Acid Profile

Many dietary recommendations advice a substantial decrease in the consumption of animal protein and a shift towards protein from plant sources.[2,4] Even though bread can be considered an important source of plant protein, the poor protein quality of wheat makes regular wheat bread (from both wholegrain or refined wheat flour) an inadequate choice to partially compensate future plant-protein requirements; especially when a substantial decrease in high-quality animal protein consumption is taken into account. The poor protein quality of wheat is mainly linked to an unbalanced amino acid composition, and to its lack of the indispensable amino acid lysine in particular.[5,12,15] The amino acid profile of RWB and HPHB was determined and is reported in Table 8. The results show that the proportions of indispensable and dispensable amino acids are very similar in both formulations. Amongst the dispensable amino acids, only the levels of glutamine/glutamic acid, proline and arginine differ substantially between RWB and HPHB. While wheat is particularly rich in glutamine, glutamic acid and proline but contains little arginine,[12] faba bean and carob show a complementary pattern for these AA.[32,88] Especially faba bean protein contains relatively small amounts of glutamine/glutamic acid and is high in arginine. This causes a decreased level of glutamine/glutamic acid and proline but an increased level of arginine in HPHB. Regarding the profile of indispensable AA in RWB and HPHB, many minor differences were observed. However, the lysine level is approximately 65 % higher in HPHB than in RWB. Also this change can be attributed to faba bean and carob proteins which are naturally richer in lysine than wheat.[12,32,88] Even though the difference of lysine contents expressed in %Protein might seem small, this difference has a big impact on the breads' overall amino acid balance and, thus, their protein quality. Especially when compared to a reference pattern of indispensable amino acids (for adults) recommended by WHO[89] and EFSA[90], the significance becomes evident. The quantity of indispensable amino acids in RWB and HPHB relative to the amino acids in the reference pattern is presented in Figure 4. The comparison with the reference pattern reveals that in both formulations lysine is the only AA, which does not reach the quantity specified as recommended intake (= 1). Therefore, lysine represents the limiting AA of the protein in RWB

Table 8 Amino acid composition of reference wheat bread and high-protein hybrid bread

Content [%Protein]	RWB	HPHB
Indispensable and conditionally indispensable AAs		
Histidine	1.92 ± 0.23	2.23 ± 0.27
Isoleucine	3.94 ± 0.48	3.77 ± 0.46
Leucine	7.33 ± 0.89	7.03 ± 0.85
Lysine	2.36 ± 0.29	3.90 ± 0.48
Cystine	1.99 ± 0.24	1.61 ± 0.19
Methionine	1.08 ± 0.13	0.97 ± 0.12
Cystine + Methionine (SAAs)	3.07 ± 0.37	2.58 ± 0.31
Phenylalanine	4.82 ± 0.59	4.46 ± 0.55
Tyrosine	2.41 ± 0.30	2.23 ± 0.27
Phenylalanine + Tyrosine (AAAs)	7.23 ± 0.88	6.69 ± 0.82
Threonine	2.70 ± 0.33	3.12 ± 0.38
Tryptophan	0.76 ± 0.48	0.78 ± 0.30
Valine	4.03 ± 0.49	4.38 ± 0.53
Total indispensable AAs	43.63 ± 5.71	43.75 ± 5.54
Dispensable AAs		
Asparagine/aspartic acid	4.13 ± 0.50	6.08 ± 0.74
Glutamine/glutamic acid	30.39 ± 3.69	25.71 ± 3.12
Glycine	3.94 ± 0.48	4.10 ± 0.50
Alanine	3.05 ± 0.37	3.40 ± 0.42
Serine	4.97 ± 0.61	4.63 ± 0.56
Proline	10.48 ± 1.27	8.11 ± 0.99
Arginine	3.74 ± 0.46	6.50 ± 0.79
Total dispensable AAs	60.69 ± 7.38	58.53 ± 7.10

Amino acid contents ± uncertainty values

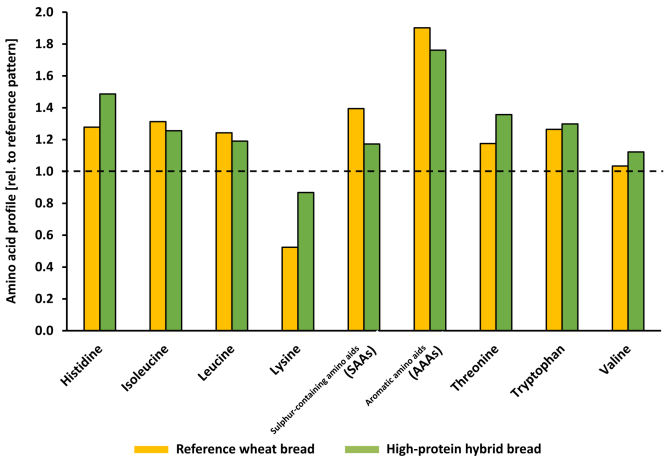

Fig. 4 Profile of indispensable amino acids of reference wheat bread and high-protein wheat bread expressed relative to the requirement pattern (WHO 2007[89]) and based on an average intake of 0.66 g protein/kg

and HPHB. The increased lysine content in HPHB (87 % of lysine in reference pattern) compared to RWB (52 % of reference pattern) leads to a much more balanced AA profile that almost covers the recommended intake of all indispensable AA. The expression of AA levels in a food protein relative to the levels in a reference protein is referred to as amino acid score (AAS). Table 9 shows an overview of AAS and limiting AAs of RWB and HPHB and the ingredients used for their production (wheat flour and HPIs). The

Table 9 Amino acid scores (AASs) for breads and their raw materials

Protein source	AAS	Limiting AAs
RWB	0.52	Lysine
HPHB	0.87	Lysine
Wheat flour*	0.57	Lysine
Faba bean flour**	0.66	SAAs
Carob germ flour*	- (1.02)***	- (Valine)***
Gluten*	0.37	Lysine

* calculated from amino acid composition; determined as for RWB and HPHB (data not shown)

** calculated from amino acid composition; determined as for RWB and HPHB and reported by Vogelsang-O'Dwyer *et al.* [32]

*** not strictly limiting (≥ 1), but represents AA with lowest level relative to reference pattern

HPHB formulation does not only have an improved AAS compared to RWB, but also in comparison to wheat flour and HPIs. The combination of the ingredients in HPHB leads to an upgrade in nutritional value of most raw materials when AAS is used to evaluate nutritional protein quality. The exception is the protein from carob germ flour, which has a nutritionally favourable AA pattern itself. Since the calculation of AAS is based on a recommended amino acid reference pattern, which considers an average intake of 0.66 g protein/kg bodyweight, this evaluation assumes that RWB or HPHB (or the ingredients) are the sole source of protein in the diet. In a real diet, proteins from other foods can potentially compensate for AA deficiencies. However, the ability of a dietary protein source to fulfil amino acid requirements on its own is regarded as an adequate approach to compare nutritional quality of proteins.

3.2.3 Protein Digestibility and Utilisation

The informative value of AASs is also limited because they do not reflect the protein's digestibility, absorption and utilisation.[91] In the present study, protein digestibility was evaluated in an *in vitro* model as well as in an *in vivo* trial with rats (Table 10). *In vitro* protein digestibility (IVPD) of RWB and HPHB was monitored after 1 h of pepsin digestion and, subsequently, 1 h of pancreatin digestion, which is indicative of the digestibility in the human digestive system. Additionally, IVPDs were measured after a medium term (3 h) and a long term (24 h) pancreatin digestion to evaluate the maximum achievable degradation of the proteins. Both the digestion mimicking gastric conditions (1 h pepsin) as well as the simulated intestinal digestion (1 h pancreatin) yielded higher ratios of degraded protein for HPHB than for RWB, indicated by significantly higher IVPD values. This suggests a slightly improved protein digestibility of HPHB, which is remarkable since legumes, in HPHB specifically faba bean and carob, are often critically discussed regarding their contents of trypsin inhibitors and an associated decrease in protein digestibility.[92] However, due to the incorporation of only 5.72 % of faba bean flour in the whole HPHB formulation (see Table 1), a substantially reduced content of trypsin inhibitors, as compared to the faba bean raw material, is expected. A detailed discussion of the trypsin inhibitor activity (TIA) in HPHB follows in chapter 3.2.4. A higher degree of protein degradation in HPHB could be explained by the higher abundance of lysine and arginine in this formulation. Trypsin, which is a predominant proteolytic enzyme in pancreatin, cleaves protein and peptide chains at the carboxyl side of these positively charged AA. Pancreatin also contains chymotrypsin, which cleaves after hydrophobic AA with bulky side chains like phenylalanine, tryptophan and tyrosine. The contents of these AA are very similar in HPHB and RWB. However, abundance of target AA for trypsin and chymotrypsin proteolysis is not the only relevant factor. Also accessibility of such AA in the three-dimensional protein structure is of high importance. This suggests that HPHB contains a higher number of AA accessible for tryptic/chymotryptic digestion. The *in vivo* protein digestibil-

Table 10 *In vitro* protein digestibility and *in vivo* nitrogen balance

Variable	RWB	HPHB
***In vitro* protein digestibility (IVPD) [%]**		
Pepsin 1 h	1.1 ± 0.4^b	2.0 ± 0.3^a
Pancreatin 1 h (short term)	14.2 ± 0.6^b	17.2 ± 0.3^a
Pancreatin 3 h (medium term)	18.4 ± 1.7^b	22.7 ± 1.2^a
Pancreatin 24 h (long term)	25.0 ± 0.0^b	31.1 ± 0.1^a
***In vivo* nitrogen balance**		
N intake [g/5 d]	$1203^b \pm 359$	1556 ± 94^a
N in faeces [mg/5 d]	138 ± 47^b	183 ± 12^a
N faecal [% N intake]	11.4 ± 1.0^a	11.8 ± 1.0^a
N in urine [mg/5 d]	766 ± 206^a	733 ± 35^a
N urinary [% N intake]	64.4 ± 3.1^a	47.1 ± 1.8^b
N digestibility [%]	88.6 ± 1.0^a	88.2 ± 1.0^a
N utilisation [%]	24.2 ± 2.7^b	41.0 ± 2.7^a
PER [g/g]	1.13 ± 0.39^b	2.13 ± 0.17^a

Means $\pm$ standard deviation with different letters in the same row were significantly different at $p < 0.05$.

Table 11 Contents of antinutritional compounds of reference wheat bread and high-protein hybrid bread, contents refer to fresh bread or dry matter as indicated

Antinutritional compound	RWB	HPHB	RWB	HPHB
	based on fresh bread		based on dry matter*	
Trypsin inhibitor activity (TIA) [TIU/mg]	n.d.	0.21 ± 0.01	n.d.	0.39 ± 0.02
Vicine [%]	n.d.	0.056 ± 0.005	n.d.	0.103 ± 0.009
Convicine [%]	n.d.	0.044 ± 0.001	n.d.	0.081 ± 0.002

Means ± standard deviation

* calculated based on moisture of fresh bread given in Table 7; for comparison purposes

ity trials performed with rats yielded several variables indicative of the breads' nutritional value (Table 10). The most important are N intake, N digestibility, N utilisation and protein efficiency ratio (PER). N intake was monitored as a reference value to calculate relative faecal and urinary N losses. N intake was significantly higher for rats which were fed the diet containing HPHB (1556 g/5 d) compared to rats with RWB diet (1203 g/5 d). Since diets were adjusted to contain the same amount of protein, this means that rats consumed significantly more of their whole diet with HPHB. It is remarkable that N intake with HPHB diet even exceeded that of rats with the control casein diet (1262 g/5 d, data not shown). This could be associated with a higher palatability of HPHB diet compared to diets containing RWB or casein. N digestibility (according to faecal N loss) was similar between the two bread formulations in this study and no significant differences were found. Although literature reports good correlations between *in vitro* and *in vivo* digestibility data,[93] some legumes have been found to reach higher digestibility in *in vitro* experiments than *in vivo*.[91] This is in agreement with the slightly higher IVPD observed for HPHB in comparison to RWB in this study. N digestibility is also used to calculate the protein digestibility corrected amino acid score (PDCAAS), which is the most commonly used indicator of nutritional protein quality. Since N digestibility of RWB and HPHB is similar, PDCAAS values follow the same trend as AAS values dicussed in the previous section. Related to its higher lysine content, HPHB reaches a PDCAAS of 0.77, which is 67 % higher than PDCAAS of RWB with 0.46. N utilisation considers N loss in both faeces and urine. Caused by a significantly lower urinary N loss of rats fed with HPHB diet, a by 69 % increased N utilisation was observed for HPHB compared to RWB. This is mainly linked to the improved AA pattern and higher content of lysine in HPHB. It has been shown that the lack of one or more essential AAs (provided by the diet and absorbed after digestion) leads to a plateau in AA retention. Other absorbed essential AA, which are present in excess of the limiting AA according to the required AA pattern, are oxidised in the blood and excreted with the urine.[89,94] In both animal and human studies, a correlation was found between level of imbalance of indispensable AA in the diet and inefficient AA utilisation leading to limited protein synthesis.[95,96] Corresponding to the higher N utilisation, also the determined PER was with 2.13 g/g significantly higher for rats with HPHB diet than for rats with RWB diet (1.13 g/g). Protein efficiency ratio is a widely used indicator of protein quality and reflects the protein's ability to fulfil AA requirements for growth (experiment performed with growing rats). An influence of overall calorie and protein intake on N utilisation and PER has been discussed.[91,94] Therefore, differences in N utilisation and PER between HPHB and RWB in this study might be partially related to the higher N intake (hence, higher calorie intake) that was observed for HPHB. While both *in vitro* and *in vivo* models have their limitations, especially regarding transferability of results to the human digestive and metabolic system, they offer a valid comparison of proteins and their nutritional quality.[93,97] Protein digestbility is a matter of the degree of hydrolysis and release of amino acids for absorption. True protein quality is considered a measure of the balance of AA which are absorbed and utilised in the human body to achieve defined metabolic actions (e.g., growth).[5,94] Even though it is unknown, which AA in particular are absorbed and utilised in which ratios, the presented results (including AA profile, IVPD, N digestibility, N utilisation and PER) conclusively suggest improved protein quality of HPHB compared to RWB.

3.2.4 Antinutritional Compounds

Trypsin inhibitors and the pyrimidine glycosides vicine and convicine are considered antinutritional compounds and their activity/contents have been determined for HPHB and RWB in this study (Table 11). It is well known that trypsin inhibitors have the ability to form a complex with the proteolytic enzyme trypsin leading to its inactivation. While this can cause adverse effects like increased pancreatic secretory activity and pancreatic hypertrophy,[24] it is often responsible for substantially reduced protein digestibility.[25] No trypsin inhibitor activity (TIA) was detected for RWB. The TIA of 0.21 TIU/mg measured for HPHB can be considered very low compared to the approximately 10 fold higher TIA in the faba bean raw material used for HPHB reported by Vogelsang-O'Dwyer *et al.*[32]. However, this reduction of TIA is mainly related to the dilution effect in the bread matrix. While heat treatment is an efficient way to inactivate trypsin inhibitors (changes in active site conformation), baking seems to be considerably less efficient than other thermal processing techniques.[92] In addition to faba bean, also carob germ flour could be a source of trypsin inhibitors in HPHB.[98,99] According to the determined IVPD of HPHB and RWB, the remaining TIA in HPHB from faba beans or carob seeds did not lead to a decreased protein digestibility of HPHB compared to RWB. The results do not allow for an interpretation whether this is due to a negligible TIA in the bread matrix or due to the overall improved protein quality compensating for TIA. The ANCs vicine and convicine are particularly relevant in foods containing faba beans.[100]

When ingested by individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, these compounds can trigger favism, which leads to acute haemolytic anaemia.[28] On average, the sum of vicine and convicine accounts for about 1 %DM in faba beans.[27,100] However, efforts in plant breeding have led to cultivars with contents of the pyrimidine glycosides as low as 0.01 - 0.02 %DM.[27] Vogelsang-O'Dwyer *et al.*[32] reported a content (vicine + convicine) of 1.25 %DM in the faba bean flour used for HPHB. While vicine and convicine were, expectedly, not detected in RWB, HPHB contains 0.056 % vicine and 0.044 % convicine (contents referring to fresh bread). In a recent study by Gallo *et al.*[101], G6PD deficient men consumed large quantities (500 g) of faba beans from a low vicine/convicine variety (0.016 % based on wet weight as ingested). It was confirmed that this level of intake was safe and favism was not triggered. Based on the outcomes from Gallo *et al.*[101] and the results of the present study, the consumption of at least 80 g of HPHB (equivalent to 2 slices of bread with a typical weight of 38 g per slice[102]) can be considered safe for individuals with G6PD deficiency. The incorporation of faba bean flour in HPHB leads to a substantial dilution of ANCs as compared to the raw material. This underlines the value of HPHB, and formulations of its kind, with regard to nutritional aspects. In theory, the separate consumption of legumes and cereals as part of a balanced diet can guarantee a balanced AA intake similar to the pattern of HPHB. But the presence of higher amounts of ANCs, which affect protein digestibility and AA bioavailability, might substantially reduce the capacity of legumes to compensate for the lack of lysine in cereals, when consumed separately.

3.2.5 Antioxidant Potential

Phenolic compounds, and specifically phenolic acids and flavonoids, exhibit many biological activities. They are well known for their antioxidant activity through which they prevent oxidative damage of biomolecules like lipids, proteins and DNA.[103] Amongst many other factors, such oxidative damages have been associated with the occurrence of both degenerative and neurodegenerative diseases such as cancer, inflammatory and cardiovascular conditions and Alzheimer's disease.[104] It has been demonstrated in epidemiological studies that high intake of foods containing high levels of compounds with antioxidant activity (e.g., whole-grain foods and legumes) can help to prevent the development of these diseases.[105–108] The total content of phenolics of RWB and HPHB was determined. Additionally, the antioxidant potential of the phenolic extracts of the breads was evaluated using ABTS, FRAP and DPPH assays. The results are presented in Table 12. The total content of phenolics is with 66.1 mg/100 g substantially higher in HPHB than in RWB with only 15 mg/100 g. Also the assays performed to determine antioxidant activity of the phenolic extracts conclusively suggest an increased antioxidant potential of HPHB than RWB. High levels of antioxidant compounds have been reported for legumes[18] and faba bean and carob in particular.[17,43,109] Therefore, they are expected to be the main contributors to the enhanced antioxidant potential of HPHB. The same trend was observed by Turfani *et al.*[23] when they evaluated antioxidant potential of breads enriched with carob flours. Also wheat is naturally rich in phenolics. But since these compounds are mainly found in the bran fraction, the antioxidant potential of breads produced from refined wheat flour is usually low.[110] Ragaee *et al.*[111] investigated the content of phenolics and antioxidant potential of refined wheat bread when wheat flour was partially replaced (30 %) by wholegrain flours from different cereals (wheat, rye, oats, barley). The incorporation of all wholegrain cereals flours increased the breads' antioxidant potential. The highest content of phenolics of approximately 70 mg/100 g was observed when wholegrain rye flour was added, which is similar to the content of phenolics reached by HPHB in the present study. Since the phenolics in a food matrix are present either free or bound to polysaccharides, a prediction whether they can exert antioxidant activity *in vivo* is difficult. Digestibility of the food, which determines bioavailability of the phenolics, is an important factor and *in vivo* antioxidant activity does not always correlate with *in vitro* data.[112] However, the results in this study clearly show higher antioxidant potential for HPHB than RWB.

Table 12 Antioxidant potential of reference wheat bread and high-protein hybrid bread, contents refer to fresh bread unless stated otherwise

Antioxidant potential	RWB	HPHB
Total phenolics [mg/100 g]	15.8 ± 0.3^b	66.1 ± 0.3^a
ABTS [mmol Trolox/100 g]	0.08 ± 0.01^b	1.02 ± 0.03^a
FRAP [mmol Fe^{2+}/100 g]	0.23 ± 0.01^b	0.77 ± 0.01^a
Antiradical activity (DPPH)		
EC_{50} [mg extract/mL]$^\times$	6.22 ± 0.18^a	1.15 ± 0.03^b

Means ± standard deviation with different letters in the same row were significantly different $p < 0.05$.

$^\times$Concentration of phenolic extract of breads able to scavenge 50 % of DPPH radicals

3.3 Sensory Characteristics

Consumer acceptance of food products is highly depending on sensory characteristics, which are in turn related to the products' technological quality. Due to its enhanced nutritional profile and qualification for the nutritional claim "high in protein",[72] HPHB can be considered a functional food. According to consumer surveys reported in literature, consumers evaluate functional foods the same way they evaluate conventional foods. Functional benefits are perceived merely as added value and cannot outweigh inferior sensory properties.[113] Sensory analysis for the two formulations in this study was performed with a trained panel using selected descriptors for bread quality (Figure 5). Reference wheat bread and HPHB reached similar scores for attributes describing taste and porosity of the crumb. Interestingly, the differences in crumb structure, which were observed in technological analyses of the breads, were not perceived by the panellists. The results for HPHB further indicate an improved crumb texture, which is often perceived as an indicator of freshness amongst consumers.[114,115] Compared to RWB, it scored significantly higher in elasticity and lower in adhesiveness. While elasticity of bread crumb is recognised as a favourable attribute, adhesiveness is often associated with stickiness and an unpleasant mouthfeel.[115] Both formulations reached similar scores in chewiness. This shows that the slightly increased initial crumb hardness for HPHB, which was

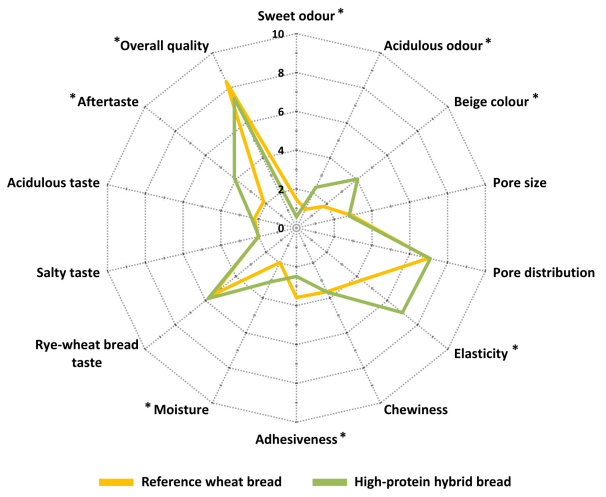

Fig. 5 Sensory characteristics of reference wheat bread and high-protein hybrid bread; asterisks * indicate attributes which showed significant differences between RWB and HPHB ($p < 0.05$)

detected in texture profile analysis (TPA), had no perceptible negative impact on the mouthfeel of the bread crumb. High-protein hybrid bread scored higher than RWB in moisture of crumb, which is considered another indicator for bread freshness and quality.[115] Significant differences have been found regarding the odour profile of the formulations. While for RWB a slight sweet and almost no acidulous odour was perceived, HPHB had no perceivable sweet odour and slightly stronger acidulous odour than RWB. In accordance with the results of instrumental crumb colour measurements, a darker/more beige colour was observed for HPHB. Also a moderate increase in aftertaste was identified in HPHB. However, the overall sensory quality was rated only slightly lower for HPHB than for RWB. This identifies HPHB as a bread formulation with adequate sensory quality when compared to RWB, suggesting high consumer acceptance. The scores of HPHB for sensory attributes like acidulous odour and beige colour suggest similarities to the typical sensory profile of sourdough bread.[116,117] Because of the popularity of sourdough bread amongst consumers, this could further contribute to a high consumer acceptance of HPHB.

4 Conclusion

A mixture of HPIs was used to partially replace wheat flour in regular wheat bread to produce a high-protein bread. The HPIs and their ratios were selected based on previous results by Hoehnel *et al.*[30] to represent both beneficial expected nutritional properties as well as adequate baking properties. In order to match the technological quality of a regular wheat bread, which was used as a reference, also three functional ingredients (psyllium, sugar, xylanase) were added. Dough and bread quality comparable to the reference wheat bread were observed for high-protein hybrid bread (HPHB); mainly mediated by the functional properties of carob and gluten protein as well as psyllium and xylanase. Additionally, a substantially enhanced nutritional profile of the proposed HPHB compared to regular wheat bread was achieved. The macronutrient composition was improved by an isocaloric replacement of refined wheat-starch by plant protein. The protein quality was improved, judging by a better AA profile, increased N utilisation and higher protein efficiency ratio. Mainly due to the dilution effect in the bread matrix, only low levels of ANCs originating from faba bean and carob were measured. Furthermore, determination of phenolics and antioxidant activity indicate high antioxidant potential for HPHB. Apart from favourable technological and nutritional characteristics, the proposed formulation also has high sensory quality which suggests high consumer acceptance. In a time in which we are looking for ways to adequately and sustainably provide enough high-quality plant protein for a future human diet, we cannot afford to focus only on meat and dairy replacement products; especially considering that these applications often require highly purified or additionally functionalised plant proteins obtained by wet-processing. In the proposed high-protein hybrid bread formulation, dry-processed protein ingredients from faba bean and carob were applied and provide a substantial amount of non-wheat protein. The increased content of plant protein with higher protein quality in HPHB and formulations of its kind, could improve the capacity of the staple food bread to cover protein needs in future plant-based diets. The results also suggest that a replacement of regular wheat bread by high-protein hybrid breads could be beneficial in currently consumed diets.

Conflicts of interest

There are no conflicts to declare.

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Abbreviations

The following abbreviations are used in this manuscript:

AA	Amino acid
SAA	Sulphur-containing amino acids
ANC	Antinutritional compound
HPI	High-protein ingredient
HPHB	High-protein hybrid bread
RWB	Reference wheat bread
LCA	Life cycle assessment
HP	High-protein
TM	Torque maximum
PMT	Peak maximum time
PV	Peak viscosity
FV	Final viscosity
V_{total}	Total gas volume produced
V_{lost}	Volume of CO_2 lost
V_{ret}	Volume of gas retained
H_M	Maximum height of dough development
SV	Specific volume
L^*crust	Lightness of crust

L^*crumb	Lightness of crumb
IVPD	*In vitro* protein digestibility
TNBS	Trinitrobenzenesulfonic acid
C	Casein
SF	Soya flour
SPI	Soya protein isolate
BW	Body weight
PER	Protein efficiency ratio
L-BAPA	N-α-benzoyl-L-arginine-4-nitroanilide
TIU	Trypsin inhibitor unit
TAU	Trypsin activity unit
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
TPC	Total phenolic content
TEAC	Trolox equivalent antioxidant capacity
FRAP	Ferric-reducing antioxidant power
DPPH	2,2-diphenyl-1-picrylhydrazyl
QDA	Quantitative descriptive analysis
ANOVA	Analysis of variance
AX	Arabinoxylans
%DM	Percentage based on dry matter
proteinE	Percentage of calories provided by protein
%E	Percentage based on energy
SFA	Saturated fatty acids
MUFA	Mono unsaturated fatty acids
PUFA	Poly unsaturated fatty acids
%Protein	Percentage based on protein
AAA	Aromatic amino acids
AAS	Amino acid score
PDCAAS	Protein digestibility corrected amino acid score
TIA	Trypsin inhibitor activity
EC_{50}	Half maximal effective concentration

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ELECTRONIC SUPPLEMENTARY INFORMATION (ESI)

for Food & Function article **"Enhancing the nutritional profile of regular wheat bread while maintaining technological quality and adequate sensory attributes"**

Andrea Hoehnel,[a] Jürgen Bez,[b] Iben Lykke Petersen,[c] Ryszard Amarowicz,[d] Jerzy Juśkiewicz,[d] Elke K. Arendt,[*a,e] and Emanuele Zannini [a]

Microbiological Shelf Life and Water Activity of Reference Wheat Bread (RWB) and High-Protein Hybrid Bread (HPHB)

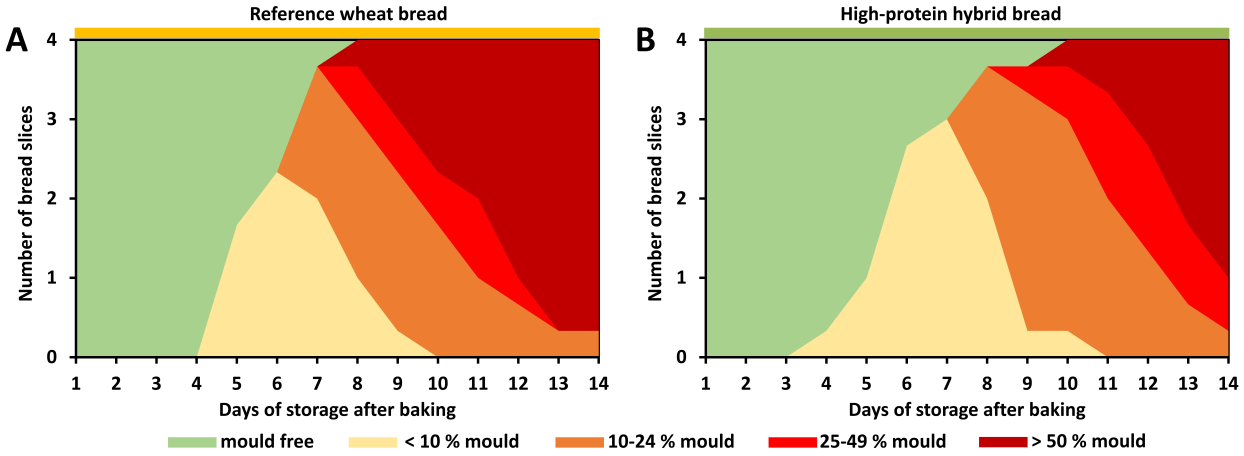

Fig. 1 Microbiological shelf life of (A) RWB and (B) HPHB as indicated by ambient air challenge test. Results represent the mean of three independently performed challenge tests.

Results and Discussion

In addition to crumb staling, the shelf life of bread is affected by microbial deterioration. While also bacteria and yeast can cause bread spoilage, a contamination with fungal spores from the bakery environment after baking is considered the most common reason.[1] Mold growth typically shows a positive correlation with water availability in the food product; the critical water activity, however, varies with fungal species, temperature and substrate.[2] Apart from an unpleasant visual experience for consumers, mould spoilage can cause the formation of off-flavours, allergenic compounds and mycotoxins, potentially even before visibility of fungal growth.[3] It also leads to a substantial amount of food waste - in UK households an estimated 20 % of bread goes to waste due to mould growth.[4,5] Therefore, susceptibility to mould deterioration represents a food safety hazard and indicator for economic loss and should be considered when bread quality is evaluated. The microbial shelf life of both bread formulations was monitored in an ambient air challenge test. The results are presented in Figure 1. A slight tendency towards earlier onset of mould growth for HPHB was observed. The results also suggest a deceleration of mould growth in HPHB represented by later onset of stages 3 - 5 (10 to >50 % of slices covered in mould). However, these tendencies cannot be considered significant differences and the experiment generally indicated a similar microbial shelf life of HPHB and RWB. This observation can be supported by very similar water activities measured for both formulations (RWB 0.945 ± 0.003, HPHB 0.943 ± 0.003).

[a] *University College Cork, School of Food and Nutritional Sciences, College Road, Ireland. Tel: +353 21 490 2064; E-mail: e.arendt@ucc.ie*
[b] *Fraunhofer Institute for Process Engineering and Packaging, 85354 Freising, Germany.*
[c] *Department of Food Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg C., Denmark.*
[d] *Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima, St. 10, 10-748 Olsztyn, Poland.*
[e] *APC Microbiome Ireland, Cork, Ireland.*

Materials and Methods used for the Determination of Microbiological Shelf Life and Water Activity

Microbiological shelf life of the breads was evaluated using an ambient mould challenge test as described by Dal Bello *et al.* [6] with some modifications. Bread loaves where sliced in a sterile manner to obtain four slices of 20 mm thickness per loaf. Instead of a treatment with conidial solutions of fungi, each slice was microbiologically challenged by exposure to the bakery ambient air for 5 min on each side. The slices were separately packed in sterile plastic bags which were heat sealed. To guarantee comparable aerobic conditions in all bags, a filter pipette tip was inserted. During a storage period of 14 days (at room temperature), mould growth was visually evaluated. Based on the percentage of slice area covered with fungal growth, slices were sorted into five categories as follows: 0 % - mould free, $<$10 % mould, 10-24 % mould, 25-49 % mould, $>$50 % mould. Four slices were monitored from each of three batches per formulation. Water activity of the fresh bread crumb was measured using a water activity meter (HygroLab, Rotronic, Basserdorf, Switzerland).

Abbreviations

The following abbreviations were used:

HPHB	High-protein hybrid bread
RWB	Reference wheat bread

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